DENSITY DISTRIBUTION OF DNA FROM PARASITIC HELMINTHS WITH SPECIAL REFERENCE TO *ASCARIS LUMBRICOIDES*

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ABSTRACT

DNA was isolated from eleven species of parasitic helminths including trematodes, cestodes, a nematode, and an acanthocephalan. The buoyant density and GC content for each was determined by analytical ultracentrifugation. These results indicated buoyant densities ranging from 1.720 to 1.697 gm/cc, corresponding to GC contents of 61% to 38%. Such results suggest the possibility that GC content (and hence AT content) may be correlated with the amount of exposure of life cycle forms to UV irradiation from sunlight, which induces $\widehat{TT}$ dimers. Highest GC content was generally observed in those species possessing freeswimming larval stages.

Analysis of buoyant density satellite DNA was accomplished by fractionation of DNA using serial preparative centrifugation in CsCl followed by analytical centrifugation. Such studies on Hymenolepidid cestodes revealed satellites with similar buoyant densities in all members of the genus that were examined. Application of this technique to somatic and germ-line tissues of *Ascaris* demonstrated that DNA from both tissues could be resolved into four density components banding approximately at 1.690, 1.696, 1.700, and 1.710 gm/cc with the main nuclear peak at 1.700.

Quantitative differences in the proportion of these components were observed between germ-line and somatic tissue DNAs as well as that of eggs and sperm. In egg DNA the peak at 1.690 was of greater magnitude that the main nuclear peak, while in sperm samples it appeared only as a minor satellite, similar to the somatic tissue DNAs. Electron microscopy of this egg satellite showed mainly circular molecules, indicating its mitochondrial origin. DNA from both egg and sperm showed an augmented peak at 1.696 in comparison to somatic tissue DNAs. In the

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latter, this component was apparent only after fractionation. These results suggest a substantial loss of this density component during chromatin elimination, without excluding the possibility of elimination from other components as well.

INTRODUCTION

Studies of DNA from a wide range of organisms have shown that species which are closely related taxonomically have similar DNA base compositions. The observation that base compositions (G + C/A + T) can be calculated from buoyant densities of DNAs (with the exception of those containing rare bases) has enabled such analyses on small quantities of DNA (Schildkraut et al., 1962). Information on the density distribution of DNA from parasitic helminths is limited to *Ascaris lumbricoides* (Bielka et al., 1968; Ward, 1971; Tobler et al., 1972); *Hymenolepis diminuta* (Carter et al., 1972; Carter and MacInnis, in preparation), and *Schistosoma mansoni* (Hillyer, 1974). In the course of our studies on various properties of DNA from parasitic helminths, we measured the buoyant densities of DNAs isolated from ten additional species and observed density satellites in most samples. Satellites are often not evident by analytical pycnography of total DNA and can be resolved only after fractionation of the DNA on preparative CsCl density gradients (McConaughy and McCarthy, 1970). Adequate amounts of test materials were not available from all species; therefore, complete analyses of satellite DNAs were limited to *H. citelli*, *H. microstoma*, and *A. lumbricoides*. The density satellites of *Ascaris* DNA prepared from germ-line as well as somatic tissue were studied in detail to determine whether chromatin elimination during development (Meyer, 1895; Bonnevie, 1902) involves a DNA component with a specific density. Bielka et al. (1968) had shown that DNA from fertilized eggs of *A. lumbricoides* banded at a density of 1.697 g/cc, with a minor satellite at 1.693 g/cc and a major one at 1.685 g/cc. DNA from the gastrula showed considerable reduction in the satellite at 1.685 g/cc. Bielka and colleagues did not correlate this reduction with nuclear chromatin elimination, but proposed a cytoplasmic origin, possibly mitochondrial, for the light satellite. However, the studies of Carter et al. (1972) showed that purified mitochondrial DNA from *Ascaris* testes is circular and bands at a density of 1.690 g/cc. Two additional conflicting reports on *Ascaris* DNA have been published (Ward, 1971; Tobler et al., 1972). These are discussed with our findings.

MATERIALS AND METHODS

Hymenolepidids and *Moniliformis dubius* were maintained in the laboratory by established methods (see MacInnis and Voge, 1970).

Adult *Ascaris lumbricoides* were collected live from the small intestines of

pigs at a Los Angeles abattoir and on arrival at the laboratory placed in 0.85% saline at 37° C for maintenance. All tissues were isolated within 4 hours of collection. After removal of a worm's viscera, the edge of a microscope slide was used to strip the muscles from the cuticle. To collect sperm, seminal vesicles were carefully removed, held on the side of a centrifuge tube with forceps and the contents drained by making a small incision on the vesicle wall. The tube contents were centrifuged and the resulting pellet was used to extract DNA. Excised *Ascaris* intestines were flushed clean by forcing 30-40 mls of saline through them with a hypodermic needle and syringe. For the extraction of DNA from the uterine wall, the terminal 3-4 cm of the uteri were used. Fertilized eggs were collected by the method of Costello (1961), and homogenized by a French pressure cell at 5000-7000 psi.

Ethanol-fixed samples of *Gyrocotyle rugosa* (from rat fish, Friday Harbor, Washington) were donated by Dr. John Simmons. *Lacystorhynchus*, *Orygmatobothrium* and *Phyllobothrium* (from *Mustelis canis*, Bodega Bay, California) were collected with the aid of Drs. Clayton Page and John Simmons. Lyophilized *Schistocephalus* (from sticklebacks, Glasgow, Scotland) was a gift from Adrian Hopkins. Lyophilized *Echinococcus multilocilaris* brood capsules (laboratory reared in cotton rats) were a gift from Dr. Dan Harlow. Ethanol-fixed *Fasciola hepatica* (from cattle, Houston, Texas) were collected with aid from Drs. Glen Harrington and John Simpson.

The DNA of these various tissues was extracted and purified by a combination of published methods. All collected samples were macerated or homogenized in a NET buffer (0.5 M NaCl, 0.1 M EDTA and 1.15 M Tris, pH 8.5). Sodium dodecyl sulfate was added to a concentration of 10 mg/ml and the samples were heated (10 minutes, 60° C). They were transferred to a 37° C water bath and pronase (preincubated: 37° C, 30 minutes) was added to give a concentration of 1 mg/ml. Following gentle shaking overnight in pronase, the samples were deproteinized once using chloroform-isoamyl alcohol (24:1 v/v) and dialyzed in 2 × SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.5). Samples were concentrated by dialysis, then treated with α-amylase (100μg/ml) and pancreatic ribonuclease A and T_1, (100μg/ml and 100 units/ml, respectively). Further purification as well as fractionation of total DNA according to buoyant density was accomplished by preparative CsCl equilibrium centrifugation. Using a preparative ultracentrifuge (Spinco Model L2) and a fixed angle 50 rotor, 200-400 μg DNA was centrifuged at 33000 rpm for 60 hours. Four-drop fractions were collected from the bottom of the gradient and diluted with water, and their absorbancies at 260 nm were measured. Appropriate fractions across the gradient were pooled and rerun in a Spinco Model E analytical ultracentrifuge (20 hours, 44000 rpm at 25° C). Buoyant densities and GC (guanine + cytosine) content were calculated by the method of Schildkraut et al. (1962), *Micrococcus lysodeikticus* DNA being used as a density marker (1.731 gm/cc). Our previous studies (Simmons

et al., 1972) demonstrated that alcohol preserved material was adequate for such studies. All chemicals used were reagent grade. Pronase, α-amylase, and ribonuclease (T_1, and pancreatic 5× Cryst) were purchased from Calbiochem.

RESULTS

GC Content

Buoyant densities of the main components of isolated DNAs and their base composition calculated from these densities are summarized in table 1. Despite the phylogenetic diversity of helminth groups studied, with the exception of *Orygmatobothrium*, no extreme differences were observed. However, a relatively higher GC content of *Orygmatobothrium*, *Schistocephalus*, *Fasciola*, *Gyrocotyle*, and *Lacystorhynchus* is apparent as compared to *Moniliformis*, *Ascaris*, and the three hymenolepidids. Singer and Ames (1970) proposed that bacterial species naturally exposed to sunlight have evolved a high GC content (and thus low adenine + thymine) as a means of avoiding genetic damage from thymine dimers formed by solar radiation. Although insufficient evidence is so far available to apply this speculation to higher organisms in general, our data appear to suggest that parasites having a free-swimming larval stage exposed to sunlight will have a relatively higher GC content of their DNA. *Echinococcus multilocularis* and the Schistosomes

TABLE I

ORGANISM	BUOYANT DENSITY OF MAIN BAND	% G + C
Orygmatobothrium sp.*	1.720	61
*Fasciola hepatica**	1.706	47
Schistocephalus sp.**	1.706	47
*Gyrocotyle rugosa**	1.705	46
*Lacystorhynchus tenuis**	1.704	45
Phyllobothrium sp.*	1.703	44
*Echinococcus multilocularis***	1.703	44
Ascaris lumbricoides	1.700	41
Moniliformis dubius	1.698	39
Hymenolepis microstoma	1.698	39
H. citelli	1.698	39
H. diminuta	1.697	38
*Schistosoma****	1.693	34

* from tissue fixed in ethanol

** lyophilized tissue; all other tissue was fresh

*** from Hillyer, 1974

appear as a possible exception. Such speculations, however, must eventually be correlated with presence or absence of the enzymes associated with repair of DNA lesions. The calculations of GC content from buoyant density must be viewed with caution, since we have no data for some species to preclude the possibility of presence of rare bases.

Analysis of Satellites: Cestodes

The microdensitometer tracings of most DNAs centrifuged to equilibrium in CsCl revealed satellite peaks. While the function of satellite DNAs observed in several organisms remains unknown, some have been shown to represent mitochondrial DNA or code for ribosomal RNA. Carter and MacInnis (unpublished) have analyzed the satellite DNAs of *H. diminuta* in some detail. In addition to the main nuclear band at 1.696 g/cc, they have observed four satellites with densities of 1.691, 1.705, 1.708, and 1.717 g/cc, respectively. The satellite at 1.691 g/cc was shown to be mitochondrial DNA (Carter et al., 1972). To compare the finding on *H. diminuta* with two closely related species, total DNAs from *H. citelli* and *H. microstoma* were each fractionated into light and heavy density components on preparative CsCl gradients. The density distribution of DNA from these fractions is shown in figures 1 and 2. The light fraction of *H. microstoma* revealed a satellite at 1.692 g/cc (similar to the DNA from *H. diminuta*). Our inability to demonstrate this peak in the light fraction of *H. citelli* does not preclude its presence. The small quantity of this satellite could be masked easily by the presence of much greater quantities of nuclear DNA, or it could have been lost in preparation of the sample. In the heavy fractions of *H. citelli* and *H. microstoma* in addition to the three satellites similar in density to those reported for *H. diminuta*, there is also a peak at 1.722 g/cc. It would be of interest to determine whether this fourth satellite is indeed absent in *H. diminuta*.

Satellites and Chromosome Diminution in *Ascaris*

Since the fractionating procedure of total DNA from the hymenolepidid species showed good resolution of satellite peaks, it was applied to DNA isolated from different tissues of *Ascaris* to determine whether chromatin eliminated from somatic cells during early cleavage can be identified as a distinct satellite.

Fertilized eggs and sperm were used for the isolation of DNA before chromatin diminution; intestinal wall, uterus, and muscle were used for samples subsequent to diminution. Analytical CsCl density gradient patterns of total DNAs from all these tissues indicated the presence of more than a single DNA component in each (figure 3). While DNAs from somatic tissues (figure 3a,b,c) gave a main band at density ca. 1.700 g/cc and only a shoulder at 1.690 g/cc, that from the egg was strikingly different (figure 3e). The major component of egg DNA banded at density 1.690 g/cc. In addition, there was a peak at 1.700 g/cc, a distinct component at 1.696 g/cc, and a heavy satellite

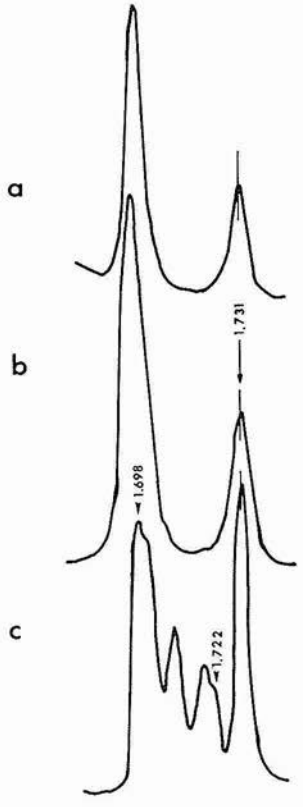

FIG. 1. ANALYTICAL PYCNOGRAPHY OF *HYMENOLEPIS CITELLI* DNA. a, total DNA; b, aliquot of pooled fractions from the light side of peak shown in a; c, aliquot of fractions from the heavy side of peak shown in a. Tracings were superimposed with the marker DNAs aligned.

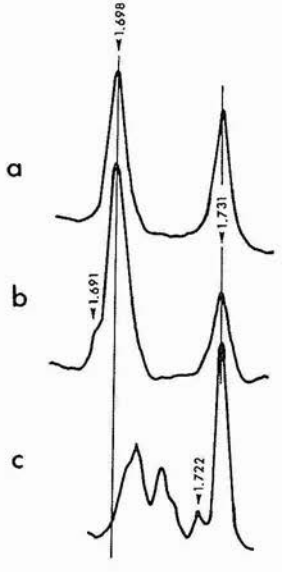

FIG. 2. ANALYTICAL PYCNOGRAPHY OF *HYMENOLEPIS MICROSTOMA* DNA. a, total DNA; b, aliquot of pooled fractions from the light side of peak shown in a; c, aliquot of fractions from the heavy side of peak shown in a. Tracings were superimposed with the marker DNA aligned.

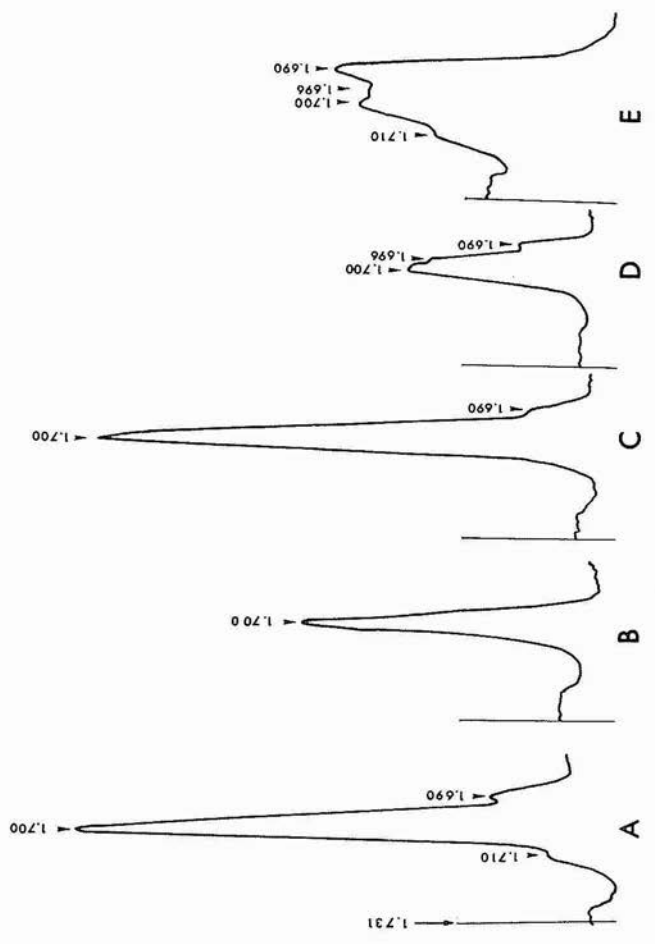

FIG. 3. MICRODENSITOMETER TRACINGS OF UV ABSORBANCE PATTERNS of *Ascaris* DNAs centrifuged to equilibrium in CsCl in a Spinco Model E analytical ultracentrifuge at 44,000 rpm for 20 hours. a, muscle DNA; b, uterine wall DNA; c, intestinal wall DNA; d, sperm DNA; e, fertilized egg DNA. Marker DNA with a density of 1.731 (arrow) was added to all samples. The line on left of each tracing indicates position of marker.

band at 1.710 g/cc. A similar heavy satellite was also evident in muscle (figure 3a). The density distribution pattern of sperm DNA was of special interest (figure 3d). Since the germinal cells supposedly arise from cell lines that do not undergo chromatin diminution, it was expected that sperm DNA would reflect a complete, undiminished DNA pattern similar to that of fertilized eggs. Unlike somatic tissue DNA, sperm samples did show the presence of a peak at 1.696 g/cc, as was also seen in the egg. However, the DNA band at 1.690 g/cc, prominent in the egg, was by comparison considerably smaller.

From the above results it could be concluded that (1) the peak at 1.700 g/cc represents the major nuclear DNA component that persists after chromatin elimination; (2) the major egg DNA peak at 1.690 g/cc is less prominent in sperm as well as somatic tissue DNAs; (3) the only apparent qualitative difference between germinal and somatic tissue DNAs is the absence of the density component at 1.696 g/cc from the latter.

Fractionated egg DNA (figure 4) confirmed the presence of four distinct density peaks as already seen in total DNA samples. The difficulty of collecting sufficient quantities of sperm limited a detailed fractionation of the DNA. However, separation of the total DNA sample into a light and heavy fraction did reveal distinct density peaks of 1.690, 1.696, and 1.702 g/cc (figure 5). Fractionation of DNA from the uterine wall gave unexpected results. What had appeared as a fairly symmetrical peak in total DNA samples (figure 3b) could now be resolved into four distinct bands (figure 6) with the same buoyant densities as seen in egg DNA. The banding pattern of total DNA from intestinal wall had clearly shown a light satellite at a density of 1.690 in addition to the main peak at 1.700 g/cc (figure 3c). Additional peaks could not be resolved in an initial attempt at fractionation (figure 7a,b,c). When the light fraction (figure 7a) was refractionated, however, a clear peak at 1.696 g/cc became evident (figure 7d). These results of fractionated uterine and intestinal wall DNAs illustrate clearly that what had appeared as a qualitative difference between total DNAs of germinal and somatic tissues is only a quantitative difference.

It would be of interest to determine whether this component at 1.696 g/cc is also present in muscle DNA. The presence of large quantities of glycogen as well as some UV-absorbing contaminants made isolation of the relatively minute quantities of pure muscle DNA a major task. A single successful preparation showed a perfectly symmetrical main band at 1.699 g/cc and two satellite bands at 1.690 and 1.710.

Discussion

We do not consider the absence of the density satellite of 1.710 g/cc from sperm and intestinal wall DNA to be significant. This could have resulted from our failure to collect enough of the heavy side of the CsCl gradients that did not show detectable absorption at 260 nm.

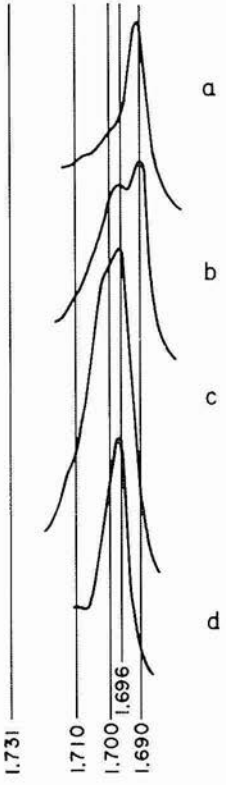

FIG. 4. ANALYTICAL PYCNOGRAPHY OF *ASCARIS* EGG DNA. About 300 μg of total native DNA was centrifuged to equilibrium in CsCl at 33,000 rpm in a fixed angle 50 rotor for 60 hours. Four-drop fractions were collected from the bottom of the tubes. Aliquots of selected, pooled fractions across the peak were then centrifuged to equilibrium in CsCl in the analytical ultracentrifuge. The tracings are arranged in sequence (a-d) starting with the lowest density fraction selected, and were superimposed with the marker DNAs aligned. Other conditions as in figure 3.

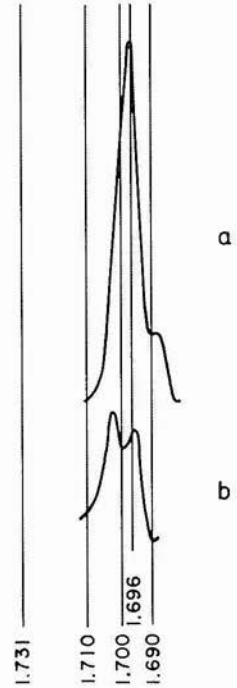

FIG. 5. ANALYTICAL PYCNOGRAPHY OF *ASCARIS* SPERM DNA. Conditions as in figure 4.

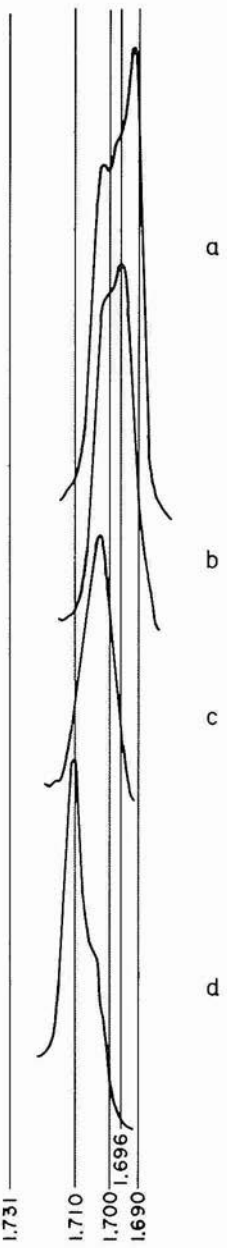

FIG. 6. ANALYTICAL PYCNOGRAPHY OF *ASCARIS* UTERINE WALL DNA. Conditions as in figure 4.

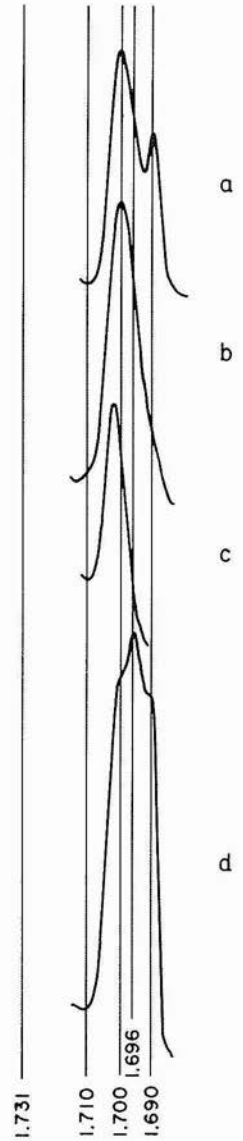

FIG. 7. ANALYTICAL PYCNOGRAPHY OF *ASCARIS* INTESTINAL WALL DNA. a-c are aliquots of selected fractions from the initial, preparative CsCl equilibrium centrifugation. 100 μg of fraction b was centrifuged to equilibrium in the preparative ultracentrifuge, and d is a light fraction from this peak analyzed with the Model E. Other conditions as in figure 4.

Because of differences of density values reported by different investigators, comparison of results from different labs becomes difficult. Small variations in buoyant densities arise from altered relative proportions of adjacent peaks in particular fractions tested (clearly demonstrated in our fractionated samples), from slight changes of the banding position of different samples within CsCl gradients, or from overloading samples (as we have often done in searches for satellite bands). We have shown that the DNA from both germinal and somatic tissues of *A. lumbricoides* can be resolved into four density components banding at 1.689-1.691, 1.694-1.696, 1.699-1.702, and 1.710-1.711 g/cc. To facilitate discussion we will refer to them by the most prevalent values obtained, i.e., density peaks of 1.690, 1.696, 1.700, and 1.710 g/cc.

Initially our results appeared different from those reported by Bielka et al. (1968); if their density values were shifted by .003 units to equate the main nuclear band values at 1.700 g/cc, however, then their egg satellite peak at 1.693 g/cc would coincide with the 1.696 g/cc band of our samples and that at 1.685 g/cc would be close to our value of 1.690 g/cc. Also, the reduction of the 1.693 and 1.685 g/cc peaks in their tracings of DNA from gastrula would be consistent with our findings that density peaks at 1.696 and 1.690 g/cc are reduced in somatic tissue DNAs. Carter et al. (1972) showed that the DNA of purified mitochondria isolated from *Ascaris* testes was circular and banded at a density of 1.690 g/cc. Electron micrographs of the egg fraction shown in figure 3a showed circular molecules in addition to linear fragments. The mitochondrial origin of this satellite would explain the reduction of this peak in sperm DNA as compared to fertilized egg DNA. Eggs of many species have been shown to have an abundance of mitochondrial DNA (Davidson, 1968); also, during fertilization the total contents of *Ascaris* sperm (including mitochondria) fuse with the oocyte cytoplasm (Foor, 1967). However, Ward (1971) reported that DNA of fertilized *Ascaris* eggs shows only two components with densities of 1.692 and 1.701 g/cc. He proposed that the light band, which is 52% of the egg DNA, represents the chromatin eliminated during cleavage, since it is absent from the gastrula and the eggs contain RNA which hybridized specifically with this light satellite. His conclusion of a nuclear origin for the light satellite would imply that our 1.690 g/cc band is a mixture of mitochondrial and nuclear DNA. We have not been able to isolate clean nuclei from *Ascaris* eggs to preclude this possibility, but our electron micrographs of this fraction indicate clearly that mitochondrial DNA contributes to the peak. In another recent publication, Tobler et al. (1972) compared DNAs isolated from *A. lumbricoides* spermatids, 4-cell stage developing eggs, and larvae. They observed a single peak at 1.697 g/cc in larval and spermatid DNA and concluded that mitochondrial DNA contributes little to spermatid DNA. DNA prepared from 4-cell stages showed an additional satellite band at 1.686 g/cc, which they consider to be of mito-

chondrial origin since electron microscopy showed circular molecules. From these observations, they concluded that eliminated DNA does not differ in density from somatic or germ-line DNA. These investigators have used ethanol precipitation and spooling during the isolation procedure, however, and report only 40% recovery of DNA using the isotope dilution method. This procedure could result in a possible differential loss of some DNA fractions, as is evident in the loss of mitochondrial DNA from their preparation of spermatid DNA. Also, they have assumed that chromatin diminution in the strain of *Ascaris* they studied does not take place until the third cleavage, following the observations of Meyer and Bonnevie made in 1895 and 1902. Opinion differs as to whether chromatin elimination starts at second or third cleavage for *Ascaris equorum* (Fogg, 1930).

Obviously, the nature of the eliminated chromatin during the cleavage of *Ascaris lumbricoides* eggs still remains a problem requiring further study. We have shown clearly an identical heterogeneity of density components within germinal and somatic tissue DNAs with only some quantitative differences. An overall reduction of chromatin from all density bands cannot be precluded with our experimental methods; however, our findings indicate that of the four DNA components the most obvious reduction is from the peak at 1.696 g/cc. The proportion of mitochondrial components to a possible nuclear DNA component at 1.690 g/cc needs reinvestigation.

ACKNOWLEDGMENTS

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